(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 May 2002 (02.05.2002)

PCT

(10) International Publication Number
WO 02/34770 A1

(51) International Patent Classification[7]: C07K 14/18, C12N 15/51, 15/62, A61K 39/29, 38/16, G01N 33/68

(21) International Application Number: PCT/GB01/04636

(22) International Filing Date: 18 October 2001 (18.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0026094.3 25 October 2000 (25.10.2000) GB

(71) Applicant *(for all designated States except US)*: **IMPERIAL COLLEGE INNOVATIONS LIMITED** [GB/GB]; Sherfield Building, Imperial College, London SW7 2AZ (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **GODKIN, Andrew, James** [GB/GB]; University Hospital of Wales, Department of Integrated Medicine, Ward A7, Cardiff CF14 4XW (GB). **THOMAS, Howard** [GB/GB]; Imperial College of Science, Technology and Medicine, St Mary's Hospital, Norfolk Place, Paddington, London W2 1PG (GB).

(74) Agent: **MILES, John, S.**; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/34770 A1

(54) Title: IDENTIFICATION OF HLA-DR RESTRICTED HCV EPITOPES

(57) Abstract: Use of a polypeptide having one or more copies of one or more epitopes selected from epitopes present in the regions between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length. A method of determining the relative prospects of a particular outcome for a subject of exposure to HCV and/or HCV infection, comprising the step of determining whether the subject has an immune response to one or more epitopes present in a region between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide.

# IDENTIFICATION OF HLA-DR RESTRICTED HCV EPITOPES

The present invention relates to methods relating to hepatitis C infection and to compounds and compositions that may be useful in such methods.

The hepatitis C virus is a single stranded RNA virus, encoded by a genome of approximately 9,500 nucleotides. It is a member of the *Flaviviridae* family, being most closely related to GBV-A, GBV-B and GBV-C, the other *Hepaciviruses* (1). The virion, which has been visualized with electron microscopy in lymphoblastoid cell cultures (2), consists of the positive strand RNA molecule, surrounded by the core (nucleocapsid) and two envelope proteins (E1,E2). In common with all members of the *Flaviviradae*, the HCV genome contains a long translational open reading frame (ORF), which encodes a single polypeptide that is cleaved co- and post-translationally by cellular and viral proteinases to yield a number of structural and non-structural proteins (3) The structural proteins comprise of core, E1 and E2. The non-structural proteins, which are not expected to be constituents of the virion, are NS2, NS3, NS4a, NS4b, NS5a, and NS5b. These have a variety of functions, necessary for viral replication, including proteinase (NS2, NS3), NTPase/helicase (NS3) and RNA-dependent RNA polymerase (RdRp) (NS5b) activities. Translation of the HCV ORF is directed via a 340 nucleotide long 5' non-coding region (5'NCR), functioning as an internal ribosome entry site (IRES). There is a second non-coding region at the 3' end of the ORF (3'NCR) which includes a poly(U) tract.

The immune response to non-cytopathic viral infections can lead to three outcomes: clearance of the virus; long term control or suppression of the virus; or uncontrolled viraemia with the possibility of progressive disease. The majority of

patients infected with hepatitis C virus or human immunodeficiency virus fall into this latter category. Hepatitis C virus (HCV) represents a more recently recognised global health problem with 4-6% of certain populations chronically infected (1).

The magnitude and type of the helper CD4+ T cell response may directly influences the outcome after acute viral infections. One of their key functions is to help an adequate cytotoxic T cell (CTL) response to develop. Identification of antigen-specific CTLs with MHC-peptide tetramers has revealed non-cytopathic viral infections such as EBV (2) or LCMV (3) lead to a massive expansion of CTL which is associated with control or elimination of the viral infection. Successful clearance of HCV also appears to be associated with a broader, more powerful CTL response during the acute infection (4, 5). Conversely, persistent viral infection in HIV (6) and HCV (7) are associated with progressive loss of CTL or low magnitude responses.

CD4+ T cells are critical for maintaining effective cytotoxic T lymphocyte responses and hence control of viraemia [reviewed in (8)]. Many of the data are derived from the mouse model of LCMV infection. In CD4-/- knockout mice high dose LCMV-WE infection or low dose LCMV-DOCILE (a more virulent strain) induces an initial CTL response which later disappears and chronic viraemia ensues. Remarkably, even transient CD4+ depletion during infection with certain virulent LCMV strains is enough to cause long term loss of functional CTLs (9).

The mechanism by which HCV evades the immune response and persists is unknown. However, there are several lines of evidence that suggest CD4+ T cells

are also critical in orchestrating clearance of HCV. Successful viral elimination has been associated with the class II major histocompatability antigen HLA-DR11 (A1*0101, B1*1101), and the reproducibility in several independent studies makes it one of the strongest infectious disease-HLA associations so far described [reviewed in (10)]. HLA class II molecules bind short pathogen derived peptide epitopes, 12 - 20 amino acids in length, and are instrumental in activating CD4+ T lymphocytes. Hence this HLA association offers a powerful argument for the central role of CD4+ T cells.

The majority of studies measuring CD4+ T cell responses have used standard proliferation assays on peripheral blood mononuclear cells (PBMCs) to recombinant viral proteins. The HCV genome of approximately 9000 bases encodes a series of proteins including the structural proteins core and envelope (E1, E2), and the non-structural proteins NS2-5 (11). Earlier studies revealed most individuals who have cleared the virus have increased responses to one or more viral proteins (7-70% response rate depending on the protein) compared to chronically infected individuals (3-40%), but the immunogenicity of different proteins seems to vary between studies (12-14). There is additional *in vitro* data suggesting a stronger CD4+ T cell proliferative response during the acute infection is more likely to lead to clearance (15), and that loss of this response is associated with recurrence of the virus (16). A recent report on the long-term follow-up of a cohort of patients who had been infected with the same strain of HCV reveals a persistent proliferative T cell response to one or more viral proteins in approximately 79% of those who have cleared the virus compared to 25% of chronically infected individuals (17).

Very few CD4+ T cell epitopes have been identified in HCV infection, in contrast to the numerous CTL epitopes described (18, 19). Identifying specific T cell epitopes allows new questions to be addressed [reviewed in (20)], such as epitope-specific T cell frequencies, their role in memory, hierarchies of response, and cytokine production (e.g. Th1 IFNγ-producing, Th2 IL4-producing or Th0 IFNγ / IL4). The difficulty in identifying CD4+ epitopes is in part due to the increased degeneracy and promiscuity of peptide binding to HLA class II compared to class I molecules (21) and the poorly understood rules of antigen processing.

We have identified HLA-DR11-restricted T cell epitopes derived from several viral proteins. We describe methods of treatment and diagnosis of patients with or at risk of HCV infection and compounds and compositions that may be useful in such methods.

A first aspect of the invention provides the use of a polypeptide having one or more copies of one or more epitopes selected from epitopes present in the regions between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length.

A further aspect of the invention provides a method of treating a patient with or at risk of hepatitis C infection wherein a polypeptide having one or more copies of one or more epitopes selected from epitopes present in the regions between amino

acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, is administered to the patient, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length.

It is considered that epitopes present in the said regions are T cell-stimulating epitopes. It is preferred that a selected epitope is a T cell-stimulating epitope.

A further aspect of the invention provides the use of a polypeptide comprising or consisting of at least eight contiguous amino acids selected from the regions between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length.

A further aspect of the invention provides a method of treating a patient with or at risk of hepatitis C infection wherein a polypeptide comprising or consisting of at least eight contiguous amino acids selected from the regions between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, is administered to the patient, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length.

As noted above, it is considered that the said regions contain T cell-stimulating epitopes. It is preferred that the said contiguous amino acids contain a T cell-stimulating epitope.

The patient is a human patient. Methods of determining the HLA type of a patient will be well known to those skilled in the art. For example, a method involving polymerase chain reaction (PCR) amplification may be used, as described, for example, in Kimura & Sasazuki (1992) 11 International Histocompatibility Workshop reference protocol for the HLA DNA typing technique, p397-419, in K Tsuji, M Aizawa and T Sasazuki (ed), HLA 1991, Oxford University Press, Oxford, United Kingdom or Nevinny-Sticke *et al* (1991) Nonradiactive HLA class II typing using polymerase chain reaction and digoxigenin-11-2'-3'-dideoxy-uridine-triphosphate labeled oligonucleotide probes. *Hum Immunol* **31**, 7-13.

It is preferred that the patient is of HLA type DR11.

It will be appreciated that the polypeptide may have one or more copies of epitope(s) from one of the said regions, ie from the region between amino acids 31-45 or 141-155 or 1207-1221 or 2268-2284 or 2941-2955 of a native HCV polypeptide. Alternatively, the polypeptide may have one or more copies of epitope(s) from one such region and additionally one or more copies of epitope(s) from one or more different such regions. Thus, the polypeptide may have or comprise one or more copies of an amino acid sequence from the region between amino acids 31-45 or 141-155 or 1207-1221 or 2268-2284 or 2941-2955 of a native HCV polypeptide, or one or more copies of amino acid sequences from one or more different such regions.

By native HCV polypeptide is meant any naturally occurring HCV polypeptide. It is preferred that the HCV polypeptide is that encoded by the viral genotype 1a, based on Simmond's classification, as known to those skilled in the art. The numbering used refers to that of the unprocessed HCV polypeptide, as used for the HCV-J isolate according to Kato *et al* (1990) *PNAS* **87**, 9524-9528, as well known to those skilled in the art. Other HCV isolates may be aligned with this sequence in order to identify the equivalent amino acid. For example, type 2 isolates may contain 4 extra codons/amino acids in the E2 sequence, whilst type 3 sequences have an insertion of 2 amino acids compared to type 1 sequences. EP 0 979, 867, for example, and references therein discusses types and subtypes of HCV polypeptide. Okamoto *et al* (1992) *J Gen Virol* **72**, 2697-2704, for example, provides a review of HCV polypeptides.

Thus, the term native HCV polypeptide includes any variation already observed within any of the described regions of HCV. In addition, mutations may be introduced, provided that the mutated sequence is immunologically cross-reactive with the unmutated sequence (ie retains the same epitope). Thus, an antibody and/or T cell response elicited by the mutated sequence should also recognise the unmutated sequence. It is preferred that a mutation is a conservative substitution, as well known to those skilled in the art. By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Mutations may be made using the methods of protein engineering and site-directed mutagenesis as well known to those skilled in the art.

The three-letter and one-letter amino acid code of the IUPAC-IUB Biochemical Nomenclature Commission is used herein. The sequence of polypeptides are

given N-terminal to C-terminal as is conventional. In particular, Xaa represents any amino acid. It is preferred that the amino acids are L-amino acids, in particular it is preferred that the amino acid residues immediately flanking (such as those within 10 to 20 residues) of the epitope-forming amino acid sequence consist of L-amino acid residues but they may be D-amino acid residues.

It is preferred that the polypeptide (ie the polypeptide administered to the patient or used in the manufacture of the medicament) does not comprise a sequence of more than 30 contiguous (ie consecutive) amino acids from a native HCV polypeptide, preferably that encoded by viral genotype 1a. It will be appreciated that the polypeptide may comprise more than one sequence of up to 30 contiguous amino acids from a native HCV polypeptide, for example a sequence of up to 30 contiguous amino acids including the amino acid sequence of region 31-45 from a native HCV polypeptide and additionally a sequence of up to 30 contiguous amino acids including the amino acid sequence of region 141-155 from a native HCV polypeptide.

It is further preferred that the polypeptide does not comprise a sequence of more than 28, 25, 22, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9 or 8 contiguous amino acids from the native HCV polypeptide. It is preferred that the polypeptide comprises or consists of a sequence or sequences of between about 8 and 18 or 20 contiguous amino acids from the native HCV polypeptide, preferably about 15 contiguous amino acids from the native HCV polypeptide.

The polypeptide may be between about 8 and 100 amino acids in length. It is further preferred that the polypeptide is between about 8 and 50, 40, 30, 20, 18, 16 or 15 amino acids in length. This may have advantages in terms of ease of

preparation and/or administration/formulation of the polypeptide when compared with longer polypeptides.

The polypeptide may alternatively be longer than 100 amino acids in length. For example the polypeptide may comprise a "carrier" portion, as known to those skilled in the art. The carrier portion may serve to aid handling, delivery or immunogenic presentation of the HCV epitope(s) of interest. For example, the polypeptide may comprise a non-HCV viral polypeptide or fragment thereof, for example a hepatitis B virus (HBV) polypeptide or fragment thereof, as discussed further below. The polypeptide may be a antiidiotypic antibody that has an epitope as defined above, as known to those skilled in the art.

Polypeptides in which one or more of the amino acid residues are chemically modified, before or after the polypeptide is synthesised, may be used providing that the function of the polypeptide, namely the production of a specific immune response *in vivo*, remains substantially unchanged. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the polypeptide from *in vivo* metabolism.

The epitope(s) (for example epitope-forming amino acid sequences) may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently antigenic themselves to obviate the use of a carrier. It may be advantageous for the polypeptide to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If

the epitope, for example epitope-forming amino acid sequence, is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the epitope-forming amino acid sequence forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. The epitope(s) as defined above in relation to the preceding aspects of the invention may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys, beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both.

Alternatively, several copies of the same or different epitope may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present). Any of the conventional ways of cross-linking polypeptides may be used, such as those generally described in O'Sullivan *et al Anal. Biochem.* (1979) **100**, 100-108. For example, the first portion may be enriched with thiol groups and the second portion reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), a heterobifunctional cross-

linking agent which incorporates a disulphide bridge between the conjugated species. Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

Further useful cross-linking agents include S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) which is a thiolating reagent for primary amines which allows deprotection of the sulphydryl group under mild conditions (Julian *et al* (1983) *Anal. Biochem.* **132**, 68), dimethylsuberimidate dihydrochloride and N,N'-o-phenylenedimaleimide.

If the polypeptide is prepared by expression of a suitable nucleotide sequence in a suitable host, then it may be advantageous to express the polypeptide as a fusion product with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.

The epitope(s) of the invention may be linked to other antigens to provide a dual effect.

A further aspect of the invention provides the use of (1) a peptidomimetic compound corresponding to a polypeptide (therapeutic polypeptide) as defined in relation to any of the preceding aspects of the invention, and/or (2) a polynucleotide capable of expressing a said polypeptide and/or (3) an antibody reactive with an epitope as defined in relation to a preceding aspect of the invention, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12. The said peptidomimetic compound, polypeptide, polynucleotide or antibody may also be useful in the manufacture of a diagnostic reagent for use in

diagnosis of a patient with or at risk of hepatitis C infection, preferably wherein the patient is of HLA type DR11 or DR12.

A further aspect of the invention provides a method of treating a patient with or at risk of hepatitis C infection, wherein the patient is administered (1) a peptidomimetic compound corresponding to a polypeptide (therapeutic polypeptide) as defined in relation to any of the preceding aspects of the invention, and/or (2) a polynucleotide capable of expressing a said polypeptide and/or (3) an antibody reactive with an epitope as defined in relation to a preceding aspect of the invention, wherein the patient is of HLA type DR11 or DR12.

The term "peptidomimetic" refers to a compound that mimics the conformation and desirable features of a particular peptide as a therapeutic agent, but that avoids the undesirable features. For example, morphine is a compound which can be orally administered, and which is a peptidomimetic of the peptide endorphin.

Therapeutic applications involving peptides are limited, due to lack of oral bioavailability and to proteolytic degradation. Typically, for example, peptides are rapidly degraded *in vivo* by exo- and endopeptidases, resulting in generally very short biological half-lives. Another deficiency of peptides as potential therapeutic agents is their lack of bioavailability via oral administration. Degradation of the peptides by proteolytic enzymes in the gastrointestinal tract is likely to be an important contributing factor. The problem is, however, more complicated because it has been recognised that even small, cyclic peptides which are not subject to rapid metabolite inactivation nevertheless exhibit poor oral bioavailability. This is likely to be due to poor transport across the intestinal membrane and rapid clearance from the blood by hepatic extraction and subsequent excretion into the intestine. These observations suggest that multiple amide bonds may interfere with

oral bioavailability. It is thought that the peptide bonds linking the amino acid residues in the peptide chain may break apart when the peptide drug is orally administered.

There are a number of different approaches to the design and synthesis of peptidomimetics. In one approach, such as disclosed by Sherman and Spatola, *J. Am. Chem. Soc.*, 112: 433 (1990), one or more amide bonds have been replaced in an essentially isoteric manner by a variety of chemical functional groups. This stepwise approach has met with some success in that active analogues have been obtained. In some instances, these analogues have been shown to possess longer biological half-lives than their naturally-occurring counterparts. Nevertheless, this approach has limitations. Successful replacement of more than one amide bond has been rare. Consequently, the resulting analogues have remained susceptible to enzymatic inactivation elsewhere in the molecule. When replacing the peptide bond it is preferred that the new linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond.

Retro-inverso peptidomimetics, in which the peptide bonds are reversed, can be synthesised by methods known in the art, for example such as those described in Mézière *et al* (1997) *J. Immunol.* **159** 3230-3237. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

In another approach, a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have been used to modify mammalian peptides. Alternatively, a presumed bioactive conformation has been stabilised by

a covalent modification, such as cyclisation or by incorporation of γ-lactam or other types of bridges. See, eg. Veber *et al*, *Proc. Natl. Acad. Sci. USA*, 75:2636 (1978) and Thursell *et al*, *Biochem. Biophys. Res. Comm.*, 111:166 (1983).

A common theme among many of the synthetic strategies has been the introduction of some cyclic moiety into a peptide-based framework. The cyclic moiety restricts the conformational space of the peptide structure and this frequently results in an increased affinity of the peptide for a particular biological receptor. An added advantage of this strategy is that the introduction of a cyclic moiety into a peptide may also result in the peptide having a diminished sensitivity to cellular peptidases.

One approach to the synthesis of cyclic stabilised peptidomimetics is ring closing metathesis (RCM). This method involves steps of synthesising a peptide precursor and contacting it with a RCM catalyst to yield a conformationally restricted peptide. Suitable peptide precursors may contain two or more unsaturated C-C bonds. The method may be carried out using solid-phase-peptide-synthesis techniques. In this embodiment, the precursor, which is anchored to a solid support, is contacted with a RCM catalyst and the product is then cleaved from the solid support to yield a conformationally restricted peptide.

By an antibody is included an antibody or other immunoglobulin, or a fragment or derivative thereof, as discussed further below.

The variable heavy ($V_H$) and variable light ($V_L$) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent

antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the $V_H$ and $V_L$ partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the $V_H$ and $V_L$ partner domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

Preferably, the antibody has an affinity for the epitope of between about $10^5.M^{-1}$ to about $10^{12}.M^{-1}$, more preferably at least $10^8.M^1$.

Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982). Chimaeric antibodies are discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium* Part 2, 792-799). Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies.

The polynucleotide capable of expressing the said polypeptide may be prepared using techniques well known to those skilled in the art and as described below. Preferably the polynucleotide is capable of expressing the said polypeptide in the patient. The said polypeptide may be expressed from any suitable polynucleotide (genetic construct) as is described below and delivered to the patient. Typically, the genetic construct which expresses the polypeptide comprises the said polypeptide coding sequence operatively linked to a promoter which can express the transcribed polynucleotide (eg mRNA) molecule in a cell of the patient, which may be translated to synthesise the said polypeptide. Suitable promoters will be known to those skilled in the art, and may include promoters for ubiquitously

expressed, for example housekeeping genes or for tissue-specific genes, depending upon where it is desired to express the said polypeptide, as discussed further below.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

Preferably, the genetic construct is adapted for delivery to a human cell.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the (dividing) cell. Targeted retroviruses are available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into pre-existing viral *env* genes (see Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a review of this and other targeted vectors for gene therapy).

It will be appreciated that retroviral methods, such as those described below, may only be suitable when the cell is a dividing cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviral DNA constructs which encode the said polypeptide may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a $neo^R$ gene). Independent

colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the target cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. The injection may be made into the area in which the target cells are present. It may be desirable to express the antigenic polypeptides in antigen presenting cells (APCs).

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes liposomes (Nässander *et al* (1992) *Cancer Res.* **52**, 646-653). Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle. It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* **6**, 1129-1144. Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-376 are also useful for delivering the

genetic construct to a cell. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cell types which over-express a cell surface protein for which antibodies are available. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the genetic construct for delivery to the target cells, for example, by forming the said liposomes in a solution of the genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected, for example intraperitoneally or directly into a site where the target cells are present.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* 40, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 3410-3414). In the first of these methods a polycation-antibody complex is

formed with the genetic construct, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalised into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs are supplied to the target cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the genetic construct, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse an area comprising target cells with the suitable delivery vehicle comprising the genetic construct for a period of time; additionally or alternatively the delivery vehicle or genetic construct can be injected directly into accessible areas comprising target cells. It may be beneficial to deliver the delivery vehicle or genetic construct systemically.

The genetic constructs can be prepared using methods well known in the art.

It will be appreciated that it may be desirable to be able to regulate temporally expression of the said polypeptide in the cell. Thus, it may be desirable that expression of the said polypeptide is directly or indirectly (see below) under the control of a promoter that may be regulated, for example by the concentration of a small molecule that may be administered to the patient when it is desired to

activate or repress (depending upon whether the small molecule effects activation or repression of the said promoter) expression of the said polypeptide. It will be appreciated that this may be of particular benefit if the expression construct is stable ie capable of expressing the said polypeptide (in the presence of any necessary regulatory molecules) in the said cell for a period of at least one week, one, two, three, four, five, six, eight months or one or more years. A preferred construct may comprise a regulatable promoter. Examples of regulatable promoters include those referred to in the following papers: Rivera *et al* (1999) *Proc Natl Acad Sci USA* **96(15)**, 8657-62 (control by rapamycin, an orally bioavailable drug, using two separate adenovirus or adeno-associated virus (AAV) vectors, one encoding an inducible human growth hormone (hGH) target gene, and the other a bipartite rapamycin-regulated transcription factor); Magari *et al* (1997) *J Clin Invest* **100(11)**, 2865-72 (control by rapamycin); Bueler (1999) *Biol Chem* **380(6)**, 613-22 (review of adeno-associated viral vectors); Bohl *et al* (1998) *Blood* **92(5)**, 1512-7 (control by doxycycline in adeno-associated vector); Abruzzese *et al* (1996) *J Mol Med* **74(7)**, 379-92 (reviews induction factors e.g., hormones, growth factors, cytokines, cytostatics, irradiation, heat shock and associated responsive elements). Tetracycline – inducible vectors may also be used. These are activated by a relatively non-toxic antibiotic that has been shown to be useful for regulating expression in mammalian cell cultures. Also, steroid-based inducers may be useful especially since the steroid receptor complex enters the nucleus where the DNA vector must be segregated prior to transcription.

This system may be further improved by regulating the expression at two levels, for example by using a tissue-specific promoter and a promoter controlled by an exogenous inducer/repressor, for example a small molecule inducer, as discussed above and known to those skilled in the art. Thus, one level of regulation may

involve linking the appropriate sequence encoding the polypeptide to an inducible promoter whilst a further level of regulation entails using a tissue-specific promoter to drive the gene encoding the requisite inducible transcription factor (which controls expression of the polypeptide from the inducible promoter). Control may further be improved by cell-type-specific targeting of the genetic construct.

A further aspect of the invention provides a chimaeric polypeptide comprising 1) a first epitope selected from epitopes present in a region between amino acids 31-45, 141-155, 1207-1221, 1245-1259, 2268-2284 and/or 2941-2955 of a native HCV polypeptide and 2) a second epitope (and optionally further epitopes) selected from said epitopes, wherein the first and second epitope are present in different said regions, and wherein the chimaeric polypeptide is not a native HCV polypeptide or fragment thereof.

Preferences in relation to the chimaeric polypeptide of the invention include appropriate preferences as indicated above in relation to the polypeptide as defined in relation to preceding aspects of the invention. It is preferred that a said selected epitope is a T cell-stimulating epitope. In a preferred embodiment, the chimaeric polypeptide comprises amino acid sequences corresponding to amino acids 31-45, 141-155, 1207-1221, 1245-1259, 2268-2284 and/or 2941-2955 of the HCV polypeptide.

A further aspect of the invention provides a peptidomimetic compound corresponding to the chimaeric polypeptide of the invention.

It will be appreciated that a chimaeric polypeptide of the invention may be used in a method or use of a previous aspect of the invention.

As known to those skilled in the art, the chimaeric polypeptide (or other polypeptide as defined in relation to an aspect of the invention) may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95%

trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

A further aspect of the invention provides a polynucleotide encoding or capable of expressing a chimaeric polypeptide of the invention. The polynucleotide of the invention may be administered to a patient, as discussed above.

The polynucleotide may be expressed in a suitable host to produce a polypeptide of the invention. Thus, the DNA encoding the chimaeric polypeptide of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the chimaeric polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued

30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the chimaeric polypeptide of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. Thus, the DNA insert may be operatively linked to an appropriate promoter. Bacterial promoters include the E.coli *lacI* and *lacZ* promoters, the T3 and T7 promoters, the *gpt* promoter, the phage λ PR and PL promoters, the *phoA* promoter and the *trp* promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters and the promoters of retroviral LTRs. Other suitable promoters will be known to the skilled artisan. The expression constructs will desirably also contain sites for transcription initiation and termination, and in the transcribed region, a ribosome binding site for translation. (Hastings *et al*, International Patent No. WO 98/16643, published 23 April 1998)

The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector and it will therefore be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence marker, with any necessary control elements, that codes for a selectable trait in the transformed cell. These markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracyclin, kanamycin or ampicillin resistance genes for culturing in *E.coli* and other bacteria. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

The polypeptide of the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulphate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography (for example using a tag fused to the polypeptide), hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

Many expression systems are known, including systems employing: bacteria (eg. *E.coli* and *Bacillus subtilis*) transformed with, for example, recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeasts (eg. *Saccaromyces cerevisiae*) transformed with, for example, yeast expression vectors;

insect cell systems transformed with, for example, viral expression vectors (eg. baculovirus) ; plant cell systems transfected with, for example viral or bacterial expression vectors; animal cell systems transfected with, for example, adenovirus expression vectors.

The vectors include a prokaryotic replicon, such as the Col E1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E.coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are: pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories (Richmond, CA, USA); p*Trc*99A, pKK223-3, pKK233-3, pDR540 and pRIT5 available from Pharmacia (Piscataway, NJ, USA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A available from Stratagene Cloning Systems (La Jolla, CA 92037, USA).

A typical mammalian cell vector plasmid is pSVL available from Pharmacia (Piscataway, NJ, USA). This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing

cells, such as COS-1 cells. An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia (Piscataway, NJ, USA). This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems (La Jolla, CA 92037, USA). Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

Methods well known to those skilled in the art can be used to construct expression vectors containing the coding sequence and, for example appropriate transcriptional or translational controls. One such method involves ligation via homopolymer tails. Another method involves ligation via cohesive ends. A further method uses synthetic molecules called linkers and adaptors. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA. A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* 239, 487-491. In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

As will be apparent to those skilled in the art, the techniques described above may also be used in the production of a polypeptide (therapeutic polypeptide) as defined in relation to earlier aspects of the invention.

A further aspect of the invention provides the use of a chimaeric polypeptide, polynucleotide or peptidomimetic compound of the invention in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection. A further aspect of the invention provides a method of treating a patient with or at risk of hepatitis C infection, wherein the patient is administered a chimaeric polypeptide, polynucleotide or peptidomimetic compound of the invention. It is preferred that the patient is of HLA type DR11 or DR12, preferably DR11.

In relation to this and previous aspects of the invention, the relevant agent may be administered as a prophylactic vaccine (or the medicament may be prepared as a prophylactic vaccine). Thus, the agent/medicament may be administered to a patient at risk of HCV infection, with the intention of immunising the patient against HCV infection. For example, healthcare workers may be at a greater risk of HCV infection (for example as a consequence of needle stick injuries) than other occupational groups. Intravenous drug users may also be at greater risk.

Alternatively, the relevant agent/medicament may be administered as a therapeutic vaccine. Thus, the agent/medicament may be administered to a patient with HCV infection. The patient may have acute or chronic HCV infection, preferably acute HCV infection. Methods of detecting HCV infection are well known to those

skilled in the art, and are mentioned in Example 1. For example, PCR may be used to detect HCV nucleic acid.

The patient may also be administered other antiviral treatment, for example is, has been, or will be administered α-interferon, as known to those skilled in the art.

A further aspect of the invention provides a pharmaceutical composition comprising a chimaeric polypeptide, polynucleotide, or peptidomimetic compound of the invention and a physiologically acceptable excipient. The composition may further comprise an component for increasing the antigenicity and/or immungenicity of the composition, for example an adjuvant and/or a cytokine.

Suitable adjuvants include Freund's complete or incomplete adjuvant, muramyl dipeptide, the "Iscoms" of EP 109 942, EP 180 564 and EP 231 039, aluminium hydroxide, saponin, DEAE-dextran, neutral oils (such as miglyol), vegetable oils (such as arachis oil), liposomes, Pluronic polyols or the Ribi adjuvant system (see, for example GB-A-2 189 141). "Pluronic" is a Registered Trade Mark.

The aforementioned compounds or a formulation thereof may be administered by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time.

Whilst it is possible for a compound to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with

the compound and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be

formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the

art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

The following delivery systems may be particularly suitable for use with polypeptide or polynucleotide active ingredients.

Polypeptides (for example) may be delivered using an injectable sustained-release drug delivery system. These are designed specifically to reduce the frequency of injections. An example of such a system is Nutropin Depot which encapsulates recombinant human growth hormone (rhGH) in biodegradable microspheres that, once injected, release rhGH slowly over a sustained period.

The polypeptide can be administered by a surgically implanted device that releases the drug directly to the required site. For example, Vitrasert releases ganciclovir directly into the eye to treat CMV retinitis. The direct application of this toxic agent to the site of disease achieves effective therapy without the drug's significant systemic side-effects.

Electroporation therapy (EPT) systems can also be employed for the administration of polypeptides. A device which delivers a pulsed electric field to cells increases the permeability of the cell membranes to the drug, resulting in a significant enhancement of intracellular drug delivery.

Polypeptides can be delivered by electroincorporation (EI). EI occurs when small particles of up to 30 microns in diameter on the surface of the skin experience electrical pulses identical or similar to those used in electroporation. In EI, these particles are driven through the stratum corneum and into deeper layers of the

skin. The particles can be loaded or coated with drugs or genes or can simply act as "bullets" that generate pores in the skin through which the drugs can enter.

An alternative method of polypeptide delivery is the ReGel injectable system that is thermo-sensitive. Below body temperature, ReGel is an injectable liquid while at body temperature it immediately forms a gel reservoir that slowly erodes and dissolves into known, safe, biodegradable polymers. The active drug is delivered over time as the biopolymers dissolve.

Polypeptides may also be delivered orally. For example, the process may employ a natural process for oral uptake of vitamin $B_{12}$ in the body to co-deliver proteins and peptides. By riding the vitamin $B_{12}$ uptake system, the protein or peptide can move through the intestinal wall. Complexes are synthesised between vitamin $B_{12}$ analogues and the drug that retain both significant affinity for intrinsic factor (IF) in the vitamin $B_{12}$ portion of the complex and significant bioactivity of the drug portion of the complex.

Polypeptides can also be introduced to cells by "Trojan peptides". These are a class of polypeptides called penetratins which have translocating properties and are capable of carrying hydrophilic compounds across the plasma membrane. This system allows direct targetting of oligopeptides to the cytoplasm and nucleus, and may be non-cell type specific and highly efficient. See Derossi *et al* (1998), *Trends Cell Biol* **8**, 84-87.

A further aspect of the invention provides a polypeptide (1207-1221 polypeptide) comprising or consisting of at least eight contiguous amino acids selected from the region comprised between amino 1207-1221 of a native HCV polypeptide, and/or

having an epitope selected from epitopes present in the said region, wherein the polypeptide is of about 8 to about 100 amino acids and/or does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide. Relevant preferences indicated in relation to earlier aspects of the invention, for example concerning the length of the polypeptide or sequences therein derived from a HCV polypeptide, also apply to this aspect of the invention. The polypeptide may be useful in relation to previous aspects of the invention.

A further aspect of the invention provides a polynucleotide capable of expressing a polypeptide (1207-1221 polypeptide) of the previous aspect of the invention. A still further aspect of the invention provides a peptidomimetic compound corresponding to the polypeptide (1207-1221 polypeptide) of the previous aspect of the invention. A further aspect of the invention provides the said polypeptide, peptidomimetic compound or polynucleotide for use in medicine.

A further aspect of the invention provides the use of the said polypeptide (1207-1221 polypeptide), peptidomimetic compound or polynucleotide in the manufacture of a medicament for the treatment of a patient with or at risk of HCV infection. A further aspect of the invention provides a pharmaceutical composition comprising a said polypeptide, peptidomimetic compound or polynucleotide and a pysiologically acceptable excipient, preferences for which are as discussed above. For example, the composition may comprise a suitable adjuvant.

In relation to all previous aspects of the invention, the polypeptide may be modified to increase its antigenicity and/or combined with a compound for

increasing its antigenicity and/or immunogenicity, as indicated above. The polypeptide may be mannosylated.

As noted above, the polypeptide may comprise a non-HCV viral polypeptide, for example a HBV polypeptide, for example a HBV core protein, as described in, for example, Clarke *et al* (1987) *Nature* **330(6146)**, 381-384 or Chambers *et al* (1996) *J Virol* **70(6)**, 4045-4052.

A further aspect of the invention provides a method of determining the relative prospects of a particular outcome for a subject of exposure to HCV and/or HCV infection, comprising the step of determining whether the subject has an immune response and/or determining the nature of the immune response, to one or more epitopes present in a region between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide. It is preferred that the patient is of HLA type DR11 or DR12, preferably DR11. The method may be used to determine the relative prospect of the patient developing chronic HCV infection. The method may comprise the step of determining whether the patient has a T cell response to the epitope(s). The method may comprise the step of determining whether the T cells produce IFNγ and/or IL10. Production of IFNγ may indicate that the patient is predisposed to clear the HCV infection, whereas production of IL10 with only low or non-detectable levels of IFNγ may indicate that the patient is predisposed to develop chronic HCV infection. The method may comprise the step of comparing responses to different epitopes. Suitable methods for investigating the immune response of the patient are described, for example, in Example 1.

A further aspect of the invention provides a method of selecting a method of treatment for a subject with or at risk of a HCV infection wherein a method according to the previous aspect of the invention is used. Thus, the method may be used to prioritise treatment with α-interferon or other agents, for example treatment according to previous aspects of the invention. The method may be used to determine which epitope(s) to administer to the patient; if a patient has a strong response to a particular epitope then it may not be necessary to administer that epitope to the patient.

The invention will now be further described by reference to the following, non-limiting, Figures and Example.

**Figure legends**

**Figure 1.** (A) *Ex vivo* responses in two patients cleared of HCV to individual peptides taken from selected pools. Peptides such as 1.2, 2.2, and 19.3/4 are clearly recognised by both individuals. Other peptides appeared to induce a response in a single patient. When compared to the *ex vivo* responses to flu (the universal epitope derived from haemagglutinin) or TT (tetanus toxoid) these frequencies of > 100 / million PBMCs are strikingly high.

Representative experiment from three short term TCLs generated to peptides 1.2, 2.2, and 19.3 showing (B) the responses are all blocked by the specific anti- HLA-DR monoclonal antibody (L243), where as the anti-HLA-DQ monoclonal (Ia3) had no effect. (C) HLA matched / mismatched BCLs were used as described in methods to identify the restricting HLA allele for these epitopes 1.2, 2.2, and 19.3.

Peptides 1.2 and 19.3 were presented purely by HLA-DR11; 2.2 was presented by HLA-DR11/12 (both serologically HLA-DR5).

**Figure 2.** The total number of TCLs generated to each of the four epitopes 1.2, 2.2,11.5 and 19.3 containing particular frequencies of cognate T cells were compared between non-viraemic patients (circles), chronically infected patients (filled circles) and HLA-DR11 healthy controls (squares). (A) IFNγ production; (B) IL10 production.

**Figure 3.** Comparison of *ex vivo* and cultured epitope-specific T cell frequencies in non-viraemic patients marked by IFNγ production. (IL4 / 10 *ex vivo* ELISPOT analysis gave negative results).

**Example 1: Characterisation of novel HLA-DR11 restricted hepatitis C virus (HCV) epitopes reveals both qualitative and quantitative differences in HCV-specific CD4+ T cell responses in chronically infected and non-viraemic patients.**

The CD4+ T cells response appears to be critical in deciding the fate of many viral infections. Clearance of HCV, which often causes persistent infection, has a strong association with the MHC class II antigen HLA-DR11 suggesting a key role for CD4+ T cells. We have identified HLA-DR11-restricted T cell epitopes derived from several viral proteins, using a CD4+ T cell epitope prediction program based on complete HLA class II-eluted pooled peptide sequence data [ (22, 23) and

additional data] and highly sensitive ELISPOT assays. This enabled us to compare specific CD4+ T cell populations in HLA-DR11 patients who have either successfully eliminated the virus or who are chronically infected, revealing fundamental differences in the qualitative and quantitative characteristics of T cell responses.

We show that irrespective of the time since viral clearance, all non-viraemic patients demonstrated strong memory CD4+ T cell responses marked by IFNγ +/- IL4/IL10 production to certain epitopes. These memory cells appeared to be divided into "resting" (Th0/1) and "effector" (Th1) memory populations. Conversely, chronically infected individuals recognised most epitopes by IL10 production, with IFNγ-producing T cells either showing low frequencies or being absent, thus demonstrating a polarity in responses between chronically infected and non-viraemic individuals. The demonstration of key T cell epitopes marked by IFNγ-producing CD4+ memory T cells in all non-viraemic individuals suggests a role for these cells in viral clearance.

The frequency and phenotype of memory cells is likely to reflect the magnitude of the initial immune response, and suggests a high frequency of IFNγ-secreting CD4+ T cells to multiple epitopes are important in clearance of HCV.

## MATERIALS AND METHODS

### Study population

Patients were diagnosed with HCV exposure by the clinical history and the presence of anti-HCV antibodies. Chronic infection was diagnosed by a positive viral specific nested PCR reaction +/- abnormal serum alanine transaminase levels. A cleared infection was diagnosed by at least three negative HCV PCRs separated by 6-12 months and normal liver function tests. The HLA typing was performed using a PCR-based method to obtain the genotype. These tests were performed by the hospital reference laboratories. Ethical committee approval (St Mary's NHS Trust) was granted for obtaining blood samples from patients.

**Peptide Synthesis**

The peptides were made according to the viral genotype 1a based on Simmond's classification. The candidate peptides 15 or 16 amino acids in length were synthesized commercially (ECHAZ micro collections, Tubingen, Germany). Each peptide was dissolved in DMSO, the majority entering solution at 50 mg / ml, and frozen at -20 °C until further use. For the majority of T cell assays, the peptides were diluted 500 fold in RPMI and sterilized via a 2 µm filter immediately prior to use. The purity of selected peptides was confirmed to be >/= 70% by HPLC and MS/MS analysis.

**Pool Sequencing and Epitope Prediction**

We used data derived from HLA-DR11 complete eluted peptide sequence data in a programme which scans proteins iteratively and predicts regions (15 amino acids in length i.e. 15mers) which are likely to be both processed and presented by this HLA molecule of interest (22, 23). Briefly, this approach uses a matrix of enrichments for each amino acid over 15 cycles derived from the pool sequence data. These data

include information not only derived from the allele-specific binding motif, but from the non-bound flanking regions reflecting antigen processing [see, for example (23)]. This matrix is used in the programme which scores each position in the peptide derived from a protein of interest, and give each iterative 15mer an average score. 15mers can then be ranked, and relatively high scoring peptides considered candidate ligands / epitopes. HLA-DR11 pool sequencing data was obtained as previously described for other alleles [(23)]. This HLA-DR11 matrix was used to scan the viral polypeptide and the top 4% of predicted ligands synthesised commercially.

**IFNγ , IL-4 and IL-10 ELISPOT assays**

The biological effect of peptides on T cells was studied in either a direct *ex vivo* assay or on cultured T cell lines using single cell cytokine release as a measure of antigen-specific effector function in a highly sensitive enzyme-linked immunospot (ELISPOT) assay (antibodies and streptavidin-alkaline phosphatase obtained from Mabtech, Nacka, Sweden). The concentrations of antibodies used and washing steps were according to the manufacturer's instructions. Briefly, 4 x $10^5$ PBMCs (extracted from heparinised whole blood by centrifugation over Lymphoprep [Nycomed, Oslo, Norway]) or 2.5 x $10^4$ T cells (obtained from TCLs grown as described below) in R-5 (RPMI with 5% heat treated fetal calf serum) were added per well of a special polymer-backed 96 well filtration plate (Millipore, Moslheim, France). The wells were pre-coated with monoclonal antibody to the cytokine of interest. The peptides were tested in duplicate wells, having been added to a final concentration of 10 µg / ml, and compared to control wells with no peptide. The plate was incubated at 37°C, 5% $CO_2$ for 18 hours for IFNγ and IL10, and 40 hours

for IL4. The plate was then developed and specific cytokine-producing T cells were enumerated at the single cell level by counting the number of spots per well minus the background. The plates were assessed by an independent observer blinded to the well contents.

**Propagation of CD4+ short term T cell lines.**

$2x10^5$ PBMCs at a concentration of 2 m cells / ml were grown with 10 μg/ml of peptide, using R-10 (RPMI plus 10% human AB serum plus antibiotics/L-glutamine supplement) and incubated in a humidified atmosphere containing 5% $CO_2$ at 37 °C. On day 3, 6 and 9 the medium was supplemented with IL2 (10% Lymphocult T, Biotest) and fresh R-10 on days 6 and 9. After day 12 the lines were washed three times in RPMI and assayed as described above. To confirm HLA restriction the anti- HLA-DR monoclonal antibody L243 and anti-DQ 1a3 were added 1 hour prior to peptide at 20 μg / ml. To confirm presentation by HLA-DR11 matched and mismatched EBV transformed B cell lines (BCLs) were used (NPH97 HLA-DRB1*1101/1502, -DQB6/B7, -DR51; NPH130 HLA-DRB1*0101/0301, -DQB2/B5, -DR52; NPH145 HLA-DRB1*1201/0301, -DQB2/6, -DR52; all kind gift from Ansar Pathan, Oxford). The BCLs +/- peptide at 100 μg / ml were incubated for three hours and washed three times in RPMI. 5 x $10^5$ cells /well were used for the ELISPOT assay with approximately 5 x $10^3$ T cells.

## RESULTS AND DISCUSSION

**Analysis of *ex vivo* responses to HLA-DR11-predicted ligands identified candidate peptides as T cell epitopes**

The ability of HLA-DR11 pool sequence data to accurately identify candidate peptides that can be processed and presented from a protein was tested on a series of known HLA-DR11 ligands (24) using the previously described epitope-prediction program (22, 23). Table 1 demonstrates this approach was highly effective in the accurate identification of peptides from a parent protein that could be presented by HLA-DR11. The candidate peptides were given scores that ranked all of them to within the top 4% of predicted ligands, with three out of eight of the tested ligands receiving scores ranking them <1%.

The entire HCV polypeptide of 3011 amino acids was scanned with the HLA-DR11 matrix and the top 4% of predicted epitopes were synthesised. For initial screening these peptides were made up into a series of pools containing five or six peptides. Fifteen HLA-DR11 positive patients were screened using an *ex vivo* IFNγ ELISPOT assay with 24 pools. Pools 1, 2, 11, 17 and 19 had a frequency of positive responses (ie >2x background) in more than 25% of individuals. The responses were dominated by individuals who had cleared the virus (data not shown). Two individuals (patient 1 and 2) who demonstrated *ex vivo* responses to these pools 1, 2, 11, 17 and 19 as well as 24, were assayed further with the individual peptides from these pools and the results are shown in Figure 1a. Certain peptides were recognised by both individuals ie peptides 1.2(core 31-45), 2.2(core 141-155), 19.3(NS5 2268-2282), 19.4(NS5 2270-2284) and 24.5(NS5 2941-2955). 19.3 and 19.4 overlap and probably represent the same epitope. Other peptides gave strong responses in a single individual e.g. 11.3 (NS3 1207-1221) and 11.5 (NS3 1245-1259). The frequencies of certain peptide-specific effector T cells was strikingly high: patient 1 responded to certain peptides, including 1.2, 2.2

**Table 1.** Summary of the ability of the HLA-DR11 matrix to identify known ligands using the prediction program. Parent proteins from which a candidate ligand originated were scanned, and each theoretical iterative 15mer (i.e. 1-15, 2-16 etc) given a calculated score. Then all the 15mers were ranked from high to low scoring, and the position in this rank of the known ligand identified. The program identified with a remarkably high degree of accuracy the shown ligands, all of which have been eluted and sequenced individually from purified HLA-DR11 molecules by a separate group (reference 24).

| protein | size | sequenced known ligand | ranked score/total number of theoretical 15mers | rank % |
|---|---|---|---|---|
| cattle serum albumin | 607 | 443-160 | 16th / 593 | 1.15 |
| cathepsin D | 412 | 390-407 | 10th / 398 | 2.5 |
| coag factor V | 2224 | 1613-1631 | 83rd / 2210 | 3.7 |
| granulin D | 594 | 559-574 | 5th / 579 | 0.86 |
| nidogen | 1247 | 457-474 | 30th / 1233 | 2.4 |
| ribophorin I | 608 | 109-126 | 4th / 594 | 0.67 |
| serotonin receptor | 422 | 359-375 | 2nd / 408 | 0.49 |
| transferrin receptor | 760 | 580-595 | 10th / 746 | 1.3 |

and 19.3 with *ex vivo* frequencies of between 96-130 specific T cells / million. To confirm that the above peptide-prediction program had accurately identify CD4+ T cells epitopes, and the HLA-restriction of these epitopes, short term TCLs were grown out from this patient to these three candidate peptides. The responses of all these lines were selectively blocked by anti-HLA-DR antibodies. Further analysis using HLA-DR11 matched / miss matched BCLs as antigen presenting cells revealed HLA-DR11/12 to be the restricting antigen, representative experiments are shown in figures 1b and 1c.

**Epitopes derived from three different viral proteins are identified by specific IFNγ-producing CD4+ T cells in all non-viraemic HLA-DR11 patients.**

Preliminary experiments on peptides 1.2, 2.2, 11.3, 11.5, 16.4, 19.3 and 24.5 using cultured TCLs revealed 1.2, 2.2, 11.5, and 19.3 to be the most frequently recognised peptides as T cell epitopes, with all the responses being HLA-DR restricted (data not shown). Hence these peptides, derived from three different viral proteins (1.2, 2.2: core 11.5: NS3; 19.3: NS5), were chosen to further characterize CD4+ T cell responses in HLA-DR11-positive HCV antibody positive patients with documented cleared infection i.e. PCR negative for HCV (non-viraemic group A, n=7) or chronic infection i.e. PCR positive (group B, n=8) 71 short term TCLs were generated as described (usually five per patient) and assayed in ELISPOT assays. 15 lines were generated in three HLA-DR11 healthy individuals and used as controls for background / primary responses.

The presence of cognate T cells in each line was identified by the frequency of epitope-specific responses enumerated by the production of IFNγ. A summary of

the overall responses to these peptides is shown in table 2. There was a striking difference between the two patient groups. All the patients in group A not only responded to a broader range of epitopes, but tended to generate multiple positive TCLs. This is in stark contrast to the paucity of responses in group B. All the non-viraemic patients responded to 2 or more epitopes compared to a weak response to two epitopes seen in only a single chronically infected patient. Six out of seven patients in group A responded strongly to a single epitope (1.2) compared to a weak response in only one out of eight in group B. The responses in group A to the other epitopes compares almost as favorably (2.2: 6/7 vs. 2/8; 11.5: 5/7 vs. 2/8; 19.3: 4/7 vs. 1/8).

To indicate the range of responses obtained, the data on all the TCLs was analysed for each group of patients in terms of the proportion of lines generated to each epitope that contained a particular frequency of responding T cells i.e. >50 - >3400 specific T cells / million (figure 2a). Even at low frequencies, there were significantly more lines generated in group A compared to B for all four epitopes. Group B only demonstrated a response clearly distinct from the control group to epitope 2.2, but few high frequency lines were generated. The strong responses in Group A were reasonably consistent across the four epitopes examined. Epitope 1.2 generated more low frequency lines, where as for epitopes 2.2, 11.5 and 19.3 even the highest frequency responses (>3400 cells / million) were present in about 10% of lines tested compared to 3% for 1.2.

These data demonstrate persistent epitope-specific memory IFNγ-producing CD4+ T cells present in the PBMCs of all individuals who have cleared the virus,

irrespective of the time lapsed since viral clearance, and the almost complete absence of IFNγ-producing T cells in chronically infected patients.

**IL10 production from epitope-specific T cells reveals differential responses between chronically infected and non-viraemic patients.**

IL10 is a cytokine produced by T cells which is thought to play an immunomodulatory role by down regulating antigen specific immune responses. IL10 may favour viral persistence by inhibiting a Th1-type cell mediated immune response (25, 26). Figure 2b summarizes the production of IL10 from TCLs generated to the above four epitopes. The magnitude of the measured responses is reduced when compared to IFNγ both in the proportion of positive lines, and the frequencies of responding T cells within the line. The patients in group A had stronger IL10 responses compared to group B to epitope 2.2 and 19.3, with an equal magnitude for 1.2.

However, the situation is reversed for epitope 11.5, with chronically infected group B patients showing a stronger response than group A. When the proportion of responding lines in group B is compared with their IFNγ production, they show markedly enhanced IL10 responses to epitopes 1.2 and 11.5, revealing that cognate epitope-specific T cells can be identified by IL10 production in these patients, all be it at a lower frequency. This balance of cytokines may reflect an initially inadequate immune response in this chronically infected group, and may reflect a strategy exploited by the virus to aid persistence. EBV encodes for an IL10-homologue which is thought to aid immune evasion. (27). Small RNA viruses like HCV lack the genomic capacity to produce immunomodulatory

cytokines, but there is some evidence that epitope variants can favour IL10 production in certain cases (28). Alternatively, the prevalence of IL10 may reflect the response of the immune system to a chronic antigenic load, switching off IFNγ production and favouring IL10 (29). This strategy might minimize immunopathology in these chronically infected individuals. Chronically infected patients produced no IL10 to 2.2, which is the only epitope to which they produced some IFNγ.

IL4 production was also measured but revealed very low frequency responses in both groups. There were slightly stronger responses in group A to peptides 2.2, 11.5, and 19.3 (% positive lines producing >200 cells / million group A vs. group B 9.7 vs. 0, 22.7 vs. 14, 6.45 vs. 0) and in group B to 1.2 ( 0 vs. 5). The patients in group A who produced IL4 in particular lines tended to also produce IFNγ / IL10 from the same lines giving a Th0 phenotype. In contrast, the IL4-producing lines in group B either produced this cytokine alone, or with IL10, suggesting a Th2 phenotype.

**The frequency of epitope-specific IFNγ-producing CD4+ T cells in short term cultured lines does not correlate in most cases to *ex vivo* frequencies suggesting distinct populations of memory CD4+ T cells.**

A key hallmark of the specific immune response is the ability to distinguish between self and non-self, and the generation of immune memory. Very little is known about the nature of T cell memory in HCV, or indeed whether a protective immunity is generated after clearing an infection. Figure 3 compares the *ex vivo* epitope-specific T cell responses to the frequencies of specific cells measured in

the short term T cell lines. There does not appear to be a direct correlation in most cases. Although in certain instances high frequency cultured responses reflect an apparently high precursor frequency measured *ex vivo*, there are many instances of very high cultured frequencies being grown out from PBMCs demonstrating very low *ex vivo* measured frequencies. This raises a number of important points. Firstly, the methods used to measure responding cells may give differing results. Some of the *ex vivo* measured frequencies shown in Figure 1 (e.g. greater than 150 epitope specific cells / million PBMCs) are greater than published precursor frequencies in HCV patients measured by T cell proliferation in limited dilution assays, even though these use whole protein as antigen and might theoretically contain multiple epitopes (14). Indeed the size of such frequencies is emphasized by comparison to other *ex vivo* measured responses e.g. in Figure 1, influenza haemagglutinin universal epitope (HA 305-320 sequence ACPKYVKQNTLKLATG) of 25 cells / million and tetanus toxoid 22 cells / million in patient 1. However, we have shown that in certain patients with a high frequency of precursor cells measured in *ex vivo* IFNγ ELISPOT assays, the epitope-specific cells will not divide in standard proliferation assays without the addition of IL2 (data not shown), suggesting a degree of T cell anergy or suppression.

The lack of correlation between the *ex vivo* and cultured frequencies suggests they are measuring distinct populations which do not necessarily reflect each other. The measured frequency of cells in the lines will be a product of the size of the precursor population and the ability of these cells to proliferate on appropriate stimulation. Hence these memory CD4+ cells appear to be divided into memory

"effector" cells (Th1 type cells that readily secrete IFNγ) and memory "resting" cells (require antigen re-stimulation and IL2 for a period before demonstrating effector functions) The realization of such distinct memory populations, and identifying the role of different memory cells in protective immunity may be important for designing future vaccine strategies [reviewed in (30)].

In summary, this paper demonstrates for the first time the effective use of HLA-derived complete eluted peptide sequence data in a computer program (22) to identify specific HLA-class II epitopes derived from HCV. In HLA-DR11 patients who have successfully cleared HCV infection, even many years prior, a powerful CD4+ T cell memory response (Th1/Th0) persists to four immunodominant epitopes in all patients. To our knowledge, no previous reports have identified measurable epitope-specific CD4+ responses in 100% of cleared patients. The presence of persistent specific memory T cells to epitopes derived from several viral proteins is likely to reflect the magnitude of the initial CD4+ T cell response when clearing the virus (31, 32). Indirectly, this strongly argues for a key role for such CD4+ T cells in viral clearance.

In contrast, chronically infected patients show a paucity of IFNγ-producing T cells responses; in these patients IL10-producing cells are present which may reflect one mechanism aiding viral persistence.

## References

1. Sharara A, C Hunt and J Hamilton. 1996. Hepatitis C. *Ann. Intern Med.*; 125(8):658-668.

2. Callan M, L Tan, N Annels, G Ogg, J Wilson, C O'Callaghan, N Steven, A McMichael and A Rickinson. 1998. Direct visualisation of antigen-specific CD8+ T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med.*; 187:1395-1402.

3. Gallimore A, A Glithero, A Godkin, A Tissot, A Pluckthun, T Elliot, H Hengartner and R Zinkernagel. 1998. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualised using soluble tetrameric MHC class I / peptide complexes. *J. Exp. Med.*; 187(9):1383-1393.

4. Cooper S, A Erickson, E Adams, J Kansopon, J Weiner, D Chien, M Houghton, P Parham and C Walker. 1999. Analysis of a successful immune response against hepatitis C virus. *Immunity*; 10(4):439-449.

5. Lechner F, D Wong, R Dunbar, R Chapman, R Chung, P Dohrenwend, G Robbins, R Phillips, P Klenerman and B Walker. 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. *J. Exp. Med.*; 191(9):1499-1512.

6. Ogg G, X Jin, S Bonhoeffer, R Dunbar, M Nowak, S Monard, J Segal, Y Cao, S Rowland-Jones, V Cerundolo and A McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of RNA. *Science*; 279:2103-2106.

7. He X, B Rehermann, F Lopez-Labrador, J Boisvert, R Cheung, J Mumm, H Wedemeyer, M Berenguer, T Wright, M Davis and H Greenberg. 1999. Quantitative analysis of hepatitis C virus-specific CD8(+) T cells in peripheral blood and liver using peptide-MHC tetramers. *Proc. Natl. Acad. Sci. USA*; 96(10):5692-5697.

8. Kalams S and B Walker. 1998. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J. Exp. Med.*; 188:2199-2204.

9. Matloubian M, R Concepcion and R Ahmed. 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J. Virol.*; 68(12):8056-8063.

10. Hill A. 1998. The immunogenetics of human infectious diseases. *Annu. Rev. Immunol.*; 16:593-617.

11. Viral hepatitis: scientific basis and clinical management. Churchill Livingstone, 1993. (Zuckerman A, Thomas H, eds).

12. Lechmann M, H Ihlenfeldt, I Braunsschweiger, G Giers, G Jung, B Matz, R Kaiser, T Sauerbruch and U Spengler. 1996. T- and B-cell responses to different hepatitis C virus antigens in patients with chronic hepatitis C infection and in healthy anti-hepatitis C virus-positive blood donors without viraemia. *Hepatology*; 24:790-795.

13. Botarelli P, M Brunetto, M Minutello, P Calvo, D Unutmaz, A Weiner, Q Choo, J Shuster, G Kuo, F Bonino, M Houghton and S Abrignani. 1993. T-lymphocyte response to hepatitis C virus in different clinical courses of infection. *Gastroenterology*; 104:580-587.

14. Ferrari C, A Valli, L Galati, A Penna, P Scaccaglia, T Giuberti, C Schianchi, G Missale, M Marin and F Fiaccadori. 1994. T cell response to structural and nonstructural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. *Hepatology*; 19:286-295.

15. Diepolder H, R Zachoval, R Hoffmann, E Wierenga, T Santantonio, M Jung, D Eichenlaub and G Pape. 1995. Possible mechanism involving T-

lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. *Lancet*; 346(8981):1006-1007.

16. Gerlach J, H Diepolder, M Jung, N Gruener, W Schraut, R Zachoval, R Hoffmann, C Schirren, T Santantonio and G Pape. 1999. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology*; 117(4):933-941.

17. Takaki A, M Wiese, G Maertens, E Depla, U Seifert, A Liebetrau, J Miller, M Manns and B Rehermann. 2000. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat. Med.*; 6(5):578-582.

18. Koziel M, D N., N Afdal, A Grakoui, C Rice, Q Choo, M Houghton and B Walker. 1995. HLA classI-restricted cytotoxic T lymphocytes specific for hepatitis C virus. *J. Clin. Inv.*; 96:2311-2321.

19. Rehermann B, K Chang, J McHutchison, R Kokka, M Houghton and F Chisari. 1996. Quantitative analysis of the peripheral blood cytotoxic T lymphocyte response in patients with chronic hepatitis C virus infection. *J. Clin. Invest.*; 98(6):1432-1440.

20. McMichael A and C O'Callaghan. 1998. A new look at T cells. *J. Exp. Med.*; 187:1367-1371.

21. Rammensee H-G. 1995. Chemistry of peptides associated with MHC class I and class II molecules. *Curr Opin in Immunol*; 7(1):85-96.

22. Davenport M, I Ho Shon and A Hill. 1995. An empirical method for the prediction of T-cell epitopes. *Immunogenetics*; 42:392-397.

23. Godkin A, M Davenport, A Willis, D Jewell and A Hill. 1998. Use of complete eluted peptide sequence data from HLA-DR and -DQ molecules to

predict T-cell epitopes, and the influence of the non-binding terminal regions of ligands in epitope selection. *J Immunol.*; 161:850-858.

24. Verreck F, A van-de-Poel, J Drijfhout, R Amons, J Coligan and F Konig. 1996. Natural peptides isolated from Gly86/Val86-containing variants of HLA-DR1, -DR11, -DR13, and -DR52. *Immunogenetics*; 43(6):392-397.

25. de Waal Malefyt R, R Ysell and J de Vries. 1993. Direct effects of IL-10 on subsets of human CD4+ T cel clones and resting T cells. *J. Immunol.*; 150:4754-4765.

26. Macatonia S, T Doherty, S Knight and A O'Garra. 1993. Differential effect of IL-10 on dendritic cell induced T cell proliferation and IFN-γ production. *J. Immunol.*; 150:3755-3765.

27. Gooding L. 1992. Virus proteins that counteract host immune responses. *Cell*; 71:5-7.

28. Wang H and D Eckels. 1999. Mutations in immunodominant T cell epitopes derived from the nonstructural 3 protein of hepatitis C virus have the potential for generating escape variants that may have important consequences for T cell recognition. *J. Immunol.*; 162(7):4177-4183.

29. Buer J, A Lanoue, A Franzke, C Garcia, H von Boehmer and A Sarukham. 1998. Interleukin 10 secretion and impaired effector function of major histocompatability complex class II-restricted T cels anergised in vivo. *J. Exp. Med*; 187(2):177-183.

30. Seder R and A Hill. 2000. Vaccines against intracellular infections requiring cellular immunity. *Nature*; 406:793-798.

31. Varga S and R Welsh. 1998. Stability of virus-specific CD4 T cell frequencies from the acute infection into long term memory. *J. Immunol.*; 161:367-374.

32. Whitmire J, K Murali-Krishna, J Altman and R Ahmed. 2000. Antiviral CD4 and CD8 T-cell memory: differences in the size of the response and activation requirements. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*; 355(1395):373-379.

CLAIMS

1. Use of a polypeptide having one or more copies of one or more epitopes selected from epitopes present in the regions between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length.

2. The use of claim 1 wherein a selected epitope is a T cell-stimulating epitope.

3. Use of a polypeptide comprising or consisting of at least eight contiguous amino acids selected from the region between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12 and wherein the polypeptide (1) does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide and/or (2) the polypeptide is of about 8 to about 100 amino acids in length.

4. The use of claim 3 wherein the said contiguous amino acids contain a T cell-stimulating epitope.

5. The use of (1) a peptidomimetic compound corresponding to a polypeptide as defined in any one of claims 1 to 4, and/or (2) a polynucleotide capable of

expressing a polypeptide as defined in any one of claims 1 to 4, and/or (3) an antibody reactive with an epitope as defined in claim 1 or 2, in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection, wherein the patient is of HLA type DR11 or DR12.

6. A chimaeric polypeptide comprising 1) a first epitope selected from epitopes present in a region between amino acids 31-45, 141-155, 1207-1221, 1245-1259, 2268-2284 and/or 2941-2955 of a native HCV polypeptide and 2) a second epitope (and optionally further epitopes) selected from said epitopes, wherein the first and second epitope are present in different said regions, and wherein the chimaeric polypeptide is not a native HCV polypeptide or fragment thereof.

7. The chimaeric polypeptide of claim 6 wherein the polypeptide comprises amino acid sequences corresponding to amino acids 31-45, 141-155, 1207-1221, 1245-1259, 2268-2284 and/or 2941-2955 of the HCV polypeptide.

8. The chimaeric polypeptide of claim 6 or 7 wherein a said selected epitope is a T cell-stimulating epitope.

9. A peptidomimetic compound corresponding to the chimaeric polypeptide of claim 6, 7 or 8.

10. A polynucleotide encoding or capable of expressing a chimaeric polypeptide according to claim 6, 7 or 8.

11. Use of a polypeptide, polynucleotide or peptidomimetic compound according to any one of claims 6 to 10 in the manufacture of a medicament for the treatment of a patient with or at risk of hepatitis C infection.

12. The use of claim 11 wherein the patient is of HLA type DRA11 or DR12.

13. The use of any one of claims 1 to 5, 11 or 12 wherein the medicament is a prophylactic vaccine.

14. The use of any one of claims 1 to 5, 11 or 12 wherein the medicament is a therapeutic vaccine.

15. A pharmaceutical composition comprising a chimaeric polypeptide, polynucleotide, or peptidomimetic compound as defined in any of claims 6 to 10 and a physiologically acceptable excipient.

16. A pharmaceutical composition according to claim 15 further comprising an component for increasing the antigenicity and/or immungenicity of the composition, for example an adjuvant and/or a cytokine.

17. A polypeptide comprising or consisting of at least eight contiguous amino acids selected from the region between amino 1207-1221 of a native HCV polypeptide, and/or having an epitope selected from epitopes present in the said region, wherein the polypeptide is of about 8 to about 100 amino acids and/or does not comprise a sequence of more than 30 contiguous amino acids from a native HCV polypeptide.

18. A peptidomimetic compound corresponding to the polypeptide of claim 17.

19. A polynucleotide capable of expressing a polypeptide according to claim 17.

20. A polypeptide, peptidomimetic compound or polynucleotide as defined in claim 17, 18 or 19 for use in medicine.

21. Use of a polypeptide, peptidomimetic compound or polynucleotide as defined in claim 20 in the manufacture of a medicament for the treatment of a patient with or at risk of HCV infection.

22. A pharmaceutical composition comprising a polypeptide, peptidomimetic compound or polynucleotide as defined in claim 20 and a physiologically acceptable excipient.

23. The use, composition or polypeptide of any of the preceding claims wherein the polypeptide is modified to increase itsantigenicity or combined with a compound for increasing its antigenicity and/or immunogenicity.

24. The use, composition or polypeptide of any of the preceding claims wherein the polypeptide is mannosylated.

25. The use, composition or polypeptide of any of the preceding claims wherein the polypeptide comprises a non-HCV viral polypeptide.

26. The use, composition or polypeptide of claim 25 wherein the non-HCV viral polypeptide is a HBV polypeptide.

27. A method of determining the relative prospects of a particular outcome for a subject of exposure to HCV and/or HCV infection, comprising the step of determining whether the subject has an immune response to one or more epitopes present in a region between amino acids 31-45, 141-155, 1207-1221, 2268-2284 and/or 2941-2955 of a native HCV polypeptide.

28. The method of claim 27 wherein the patient is of HLA type DR11 or DR12.

29. The method according to claim 27 or 28 wherein the outcome is chronic infection.

30. A method of selecting a method of treatment for a subject with or at risk of a HCV infection wherein a method according to any one of claims 27 to 29 is used.

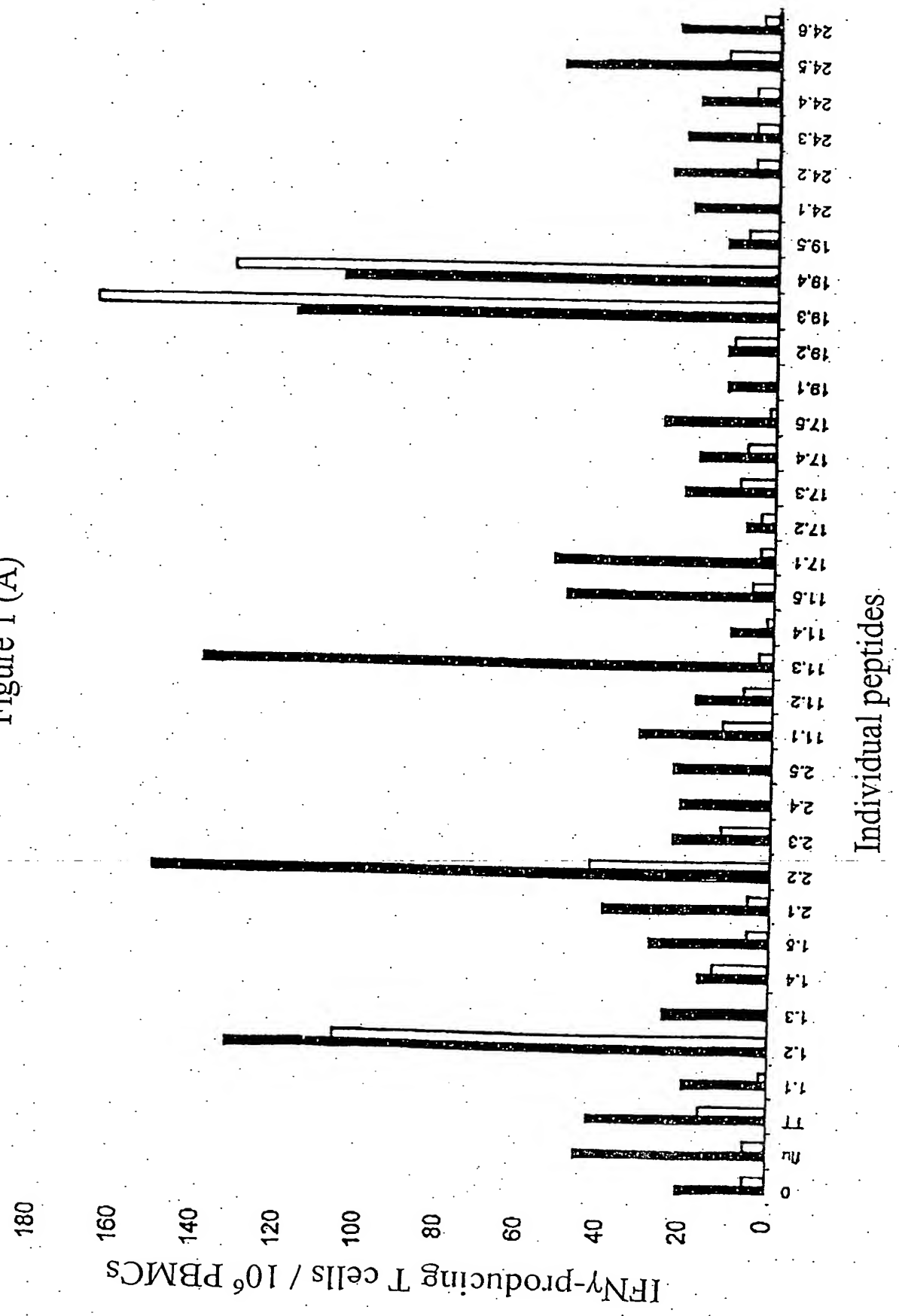


Figure 1 (A)

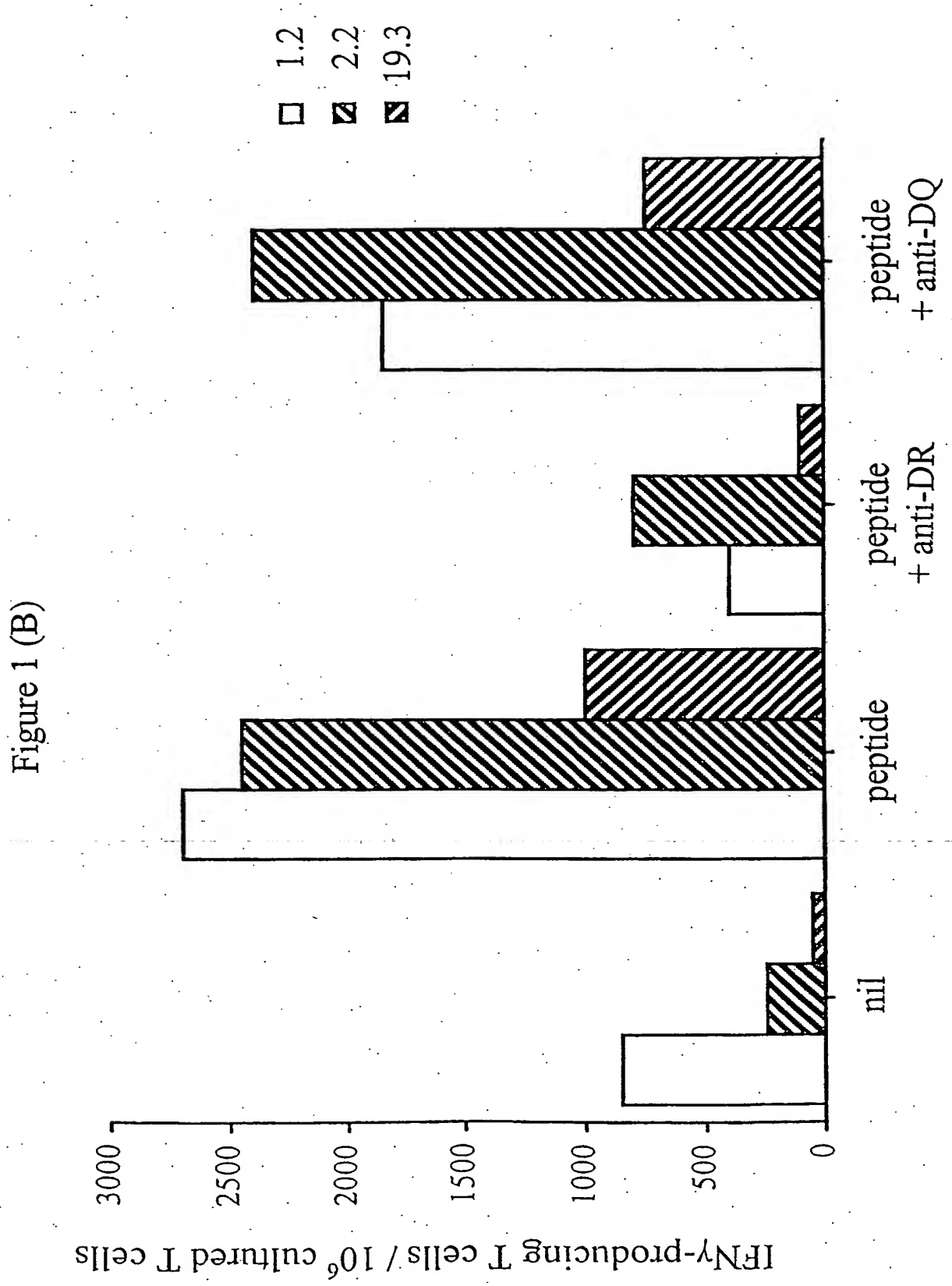


Figure 1 (B)

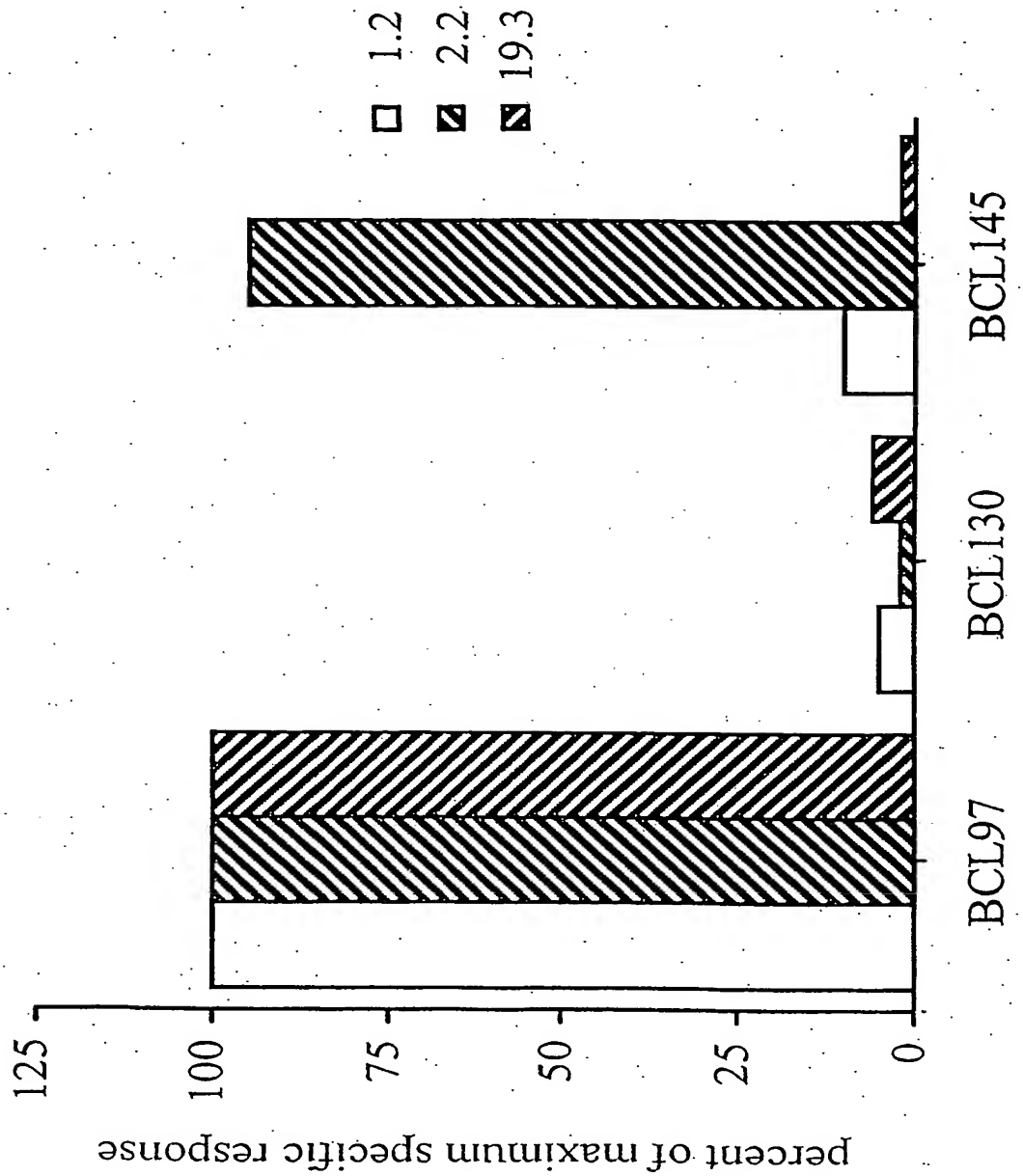


Figure 1 (C)

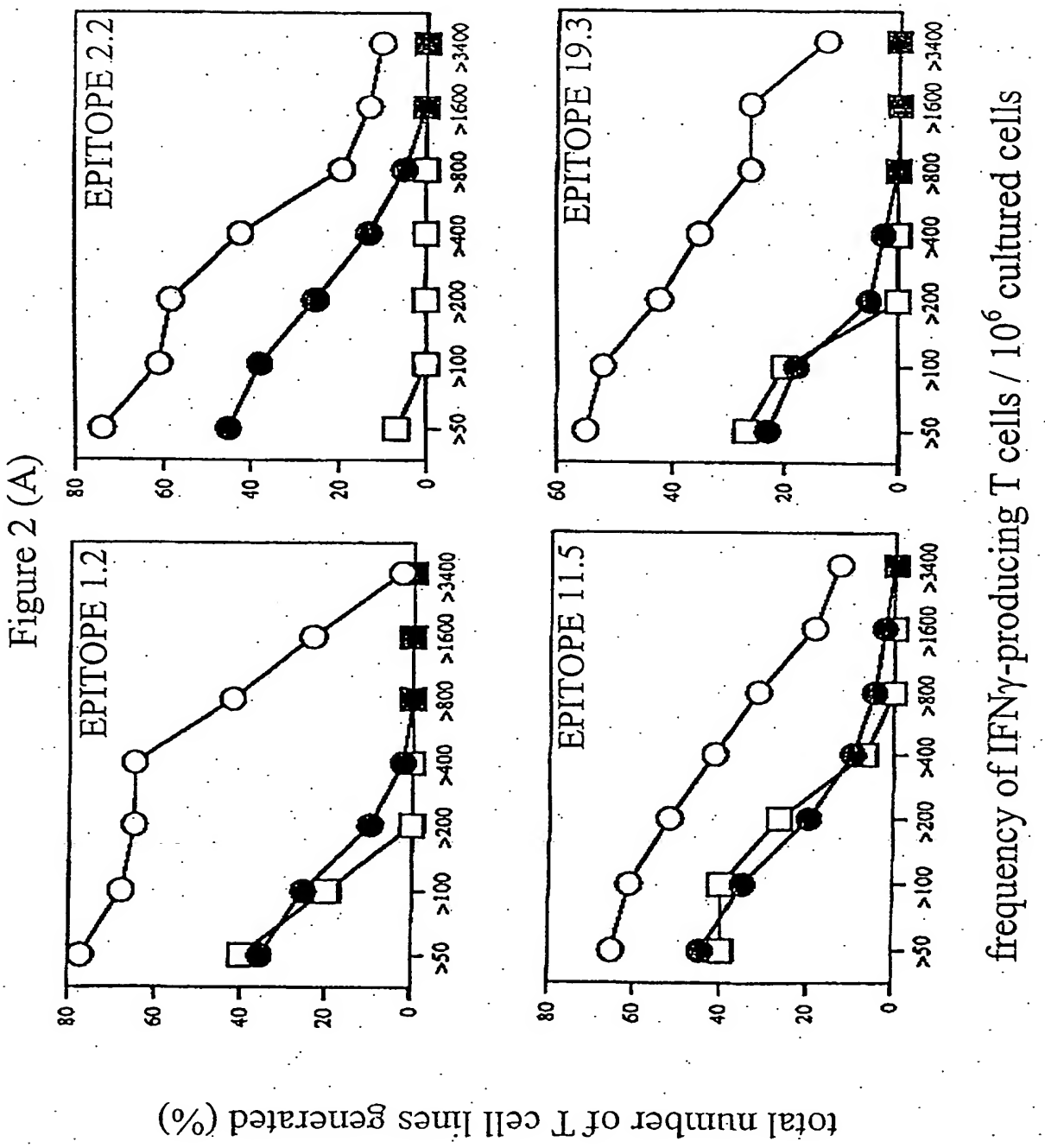


Figure 2 (A)

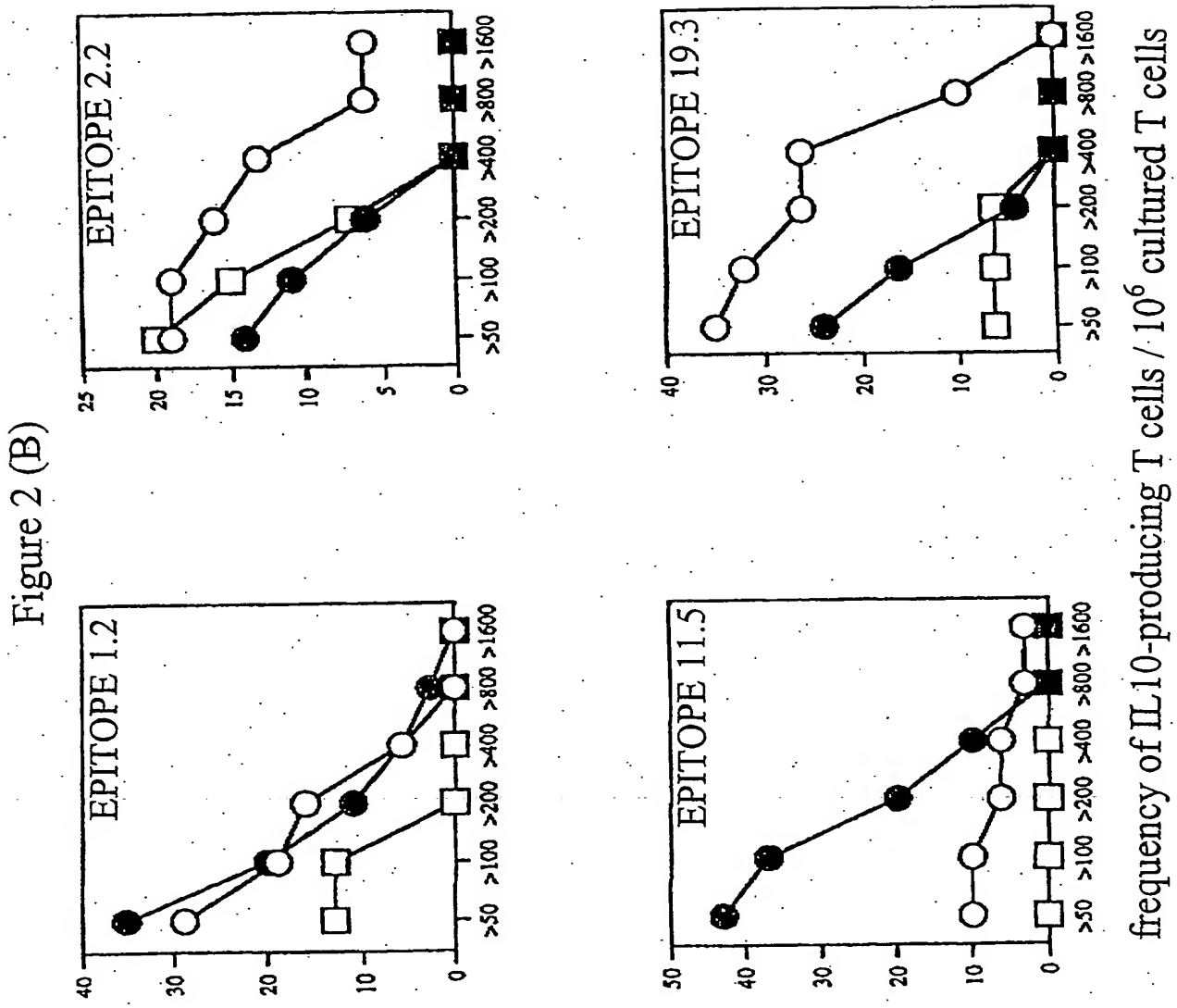


Figure 2 (B)

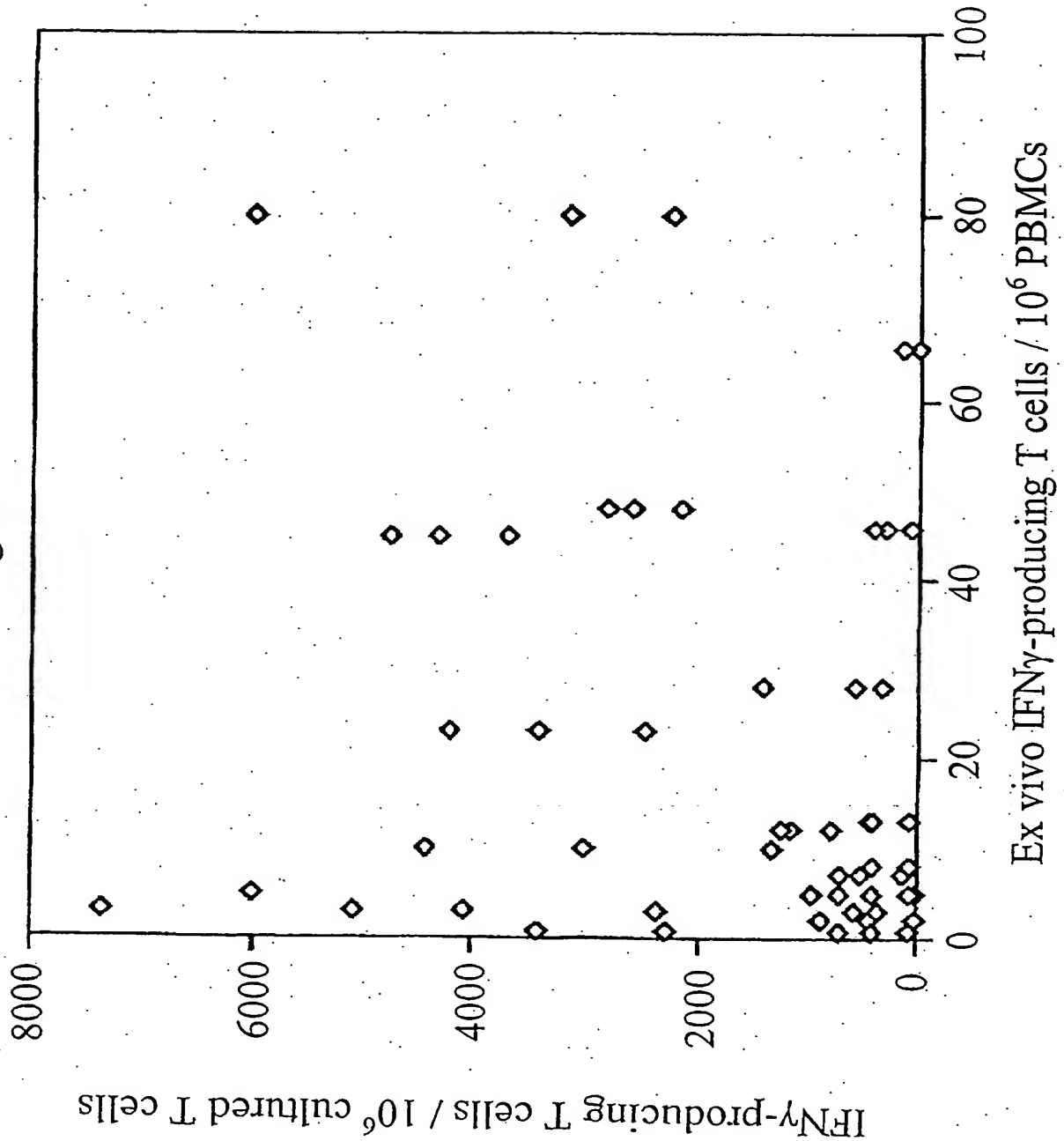


Figure 3

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/04636

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/18 C12N15/51 C12N15/62 A61K39/29 A61K38/16
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | GODKIN ET AL.: "Use of complete eluted peptide sequence data from HLA-DR and -DQ molecules to predict T-cell epitopes, and the influence of the non-binding terminal regions of ligands in epitope selection" JOURNAL OF IMMUNOLOGY, vol. 161, 1998, pages 850-858, XP002188420 cited in the application the whole document<br>---<br>-/-- | 1-30 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 28 January 2002 | 25/02/2002 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Dumont, E |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No |
|---|---|---|
| Y | THURSZ MARK ET AL: "Influence of MHC class II genotype on outcome of infection with hepatitis C virus." LANCET (NORTH AMERICAN EDITION)., vol. 354, no. 9196, 18 December 1999 (1999-12-18), pages 2119-2124, XP004263267 ISSN: 0099-5355 the whole document page 2123, paragraph 1 page 2123, last paragraph | 1-30 |
| Y | LAMONACA VINCENZO ET AL: "Conserved hepatitis C virus sequences are highly immunogenic for CD4+ T cells: Implications for vaccine development." HEPATOLOGY, vol. 30, no. 4, October 1999 (1999-10), pages 1088-1098, XP002188421 ISSN: 0270-9139 the whole document | 1-30 |
| Y | MINTON E J ET AL: "Association between MHC class II alleles and clearance of circulating hepatitis C virus." JOURNAL OF INFECTIOUS DISEASES, vol. 178, no. 1, July 1998 (1998-07), pages 39-44, XP002188422 ISSN: 0022-1899 abstract page 42, column 2, line 16,17 -page 43, column 1, paragraph 3 | 1-30 |
| A | WO 95 12677 A (DELEYS ROBERT ;INNOGENETICS NV (BE); MAERTENS GEERT (BE); LEROUX R) 11 May 1995 (1995-05-11) page 2, paragraph 3 -page 5, line 7; claims 1,23,27,32; example 4 page 27, last paragraph page 34, last paragraph -page 35, paragraph 1 page 25, line 12 - line 28 | 1-30 |
| A | WO 95 27733 A (SHIRAI MUTSUNORI ;FEINSTONE STEPHEN (US); US HEALTH (US); BERZOFSK) 19 October 1995 (1995-10-19) page 15, line 10 - line 18; claims 1-23; figure 1; example 1 | 1-30 |

-/--

International Application No
PCT/GB 01/04636

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | GODKIN ANDREW ET AL: "Characterization of novel HLA-DR11-restricted HCV epitopes reveals both qualitative and quantitative differences in HCV-specific CD4+ T cell responses in chronically infected and non-viremic patients." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 31, no. 5, May 2001 (2001-05), pages 1438-1446, XP001038659 ISSN: 0014-2980 the whole document | 1-30 |
| P,Y | WO 01 21189 A (CELIS ESTEBAN ;GREY HOWARD M (US); SIDNEY JOHN (US); CHESNUT ROBER) 29 March 2001 (2001-03-29) page 5, line 5 -page 7, line 12 page 20, line 28 -page 21, line 15 page 34, line 21 -page 35, line 2; tables III,V | 1-30 |

1

INTERNATIONAL SEARCH REPORT

Application No
PCT/GB 01/04636

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| WO 9512677 | A | 11-05-1995 | AU | 698878 B2 | 12-11-1998 |
| | | | AU | 7993294 A | 23-05-1995 |
| | | | CA | 2175692 A1 | 11-05-1995 |
| | | | WO | 9512677 A2 | 11-05-1995 |
| | | | EP | 0725824 A1 | 14-08-1996 |
| | | | EP | 0992580 A2 | 12-04-2000 |
| | | | EP | 0992581 A2 | 12-04-2000 |
| | | | EP | 0979867 A2 | 16-02-2000 |
| | | | JP | 9504534 T | 06-05-1997 |
| WO 9527733 | A | 19-10-1995 | AT | 193893 T | 15-06-2000 |
| | | | AU | 697171 B2 | 01-10-1998 |
| | | | AU | 2274895 A | 30-10-1995 |
| | | | CA | 2187299 A1 | 19-10-1995 |
| | | | DE | 69517521 D1 | 20-07-2000 |
| | | | DE | 69517521 T2 | 08-03-2001 |
| | | | EP | 0754193 A1 | 22-01-1997 |
| | | | JP | 10503473 T | 31-03-1998 |
| | | | WO | 9527733 A1 | 19-10-1995 |
| WO 0121189 | A | 29-03-2001 | AU | 6226100 A | 24-04-2001 |
| | | | WO | 0121189 A1 | 29-03-2001 |